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Maternal androgens in egg yolks: relation with sex, incubation time and embryonic growth

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Abstract

Hormones of maternal origin are known to be transferred to the egg yolks of oviparous species. Several studies have shown that within and between clutch variation of maternal androgens may be adaptively tuned. Moreover, it has recently been hypothesized that sex steroids of maternal origin may play a role in adaptive sex ratio manipulation. For sex determination the eggs have to be incubated to allow the germinal disc to grow and thus extract sufficient DNA. This means that yolk hormone levels are determined after a number of days of incubation and this may hamper interpretation of the data. If yolk utilization or embryonic hormone production are influenced by the sex of the embryo, differences in hormone content at a certain stage of incubation do not reflect the mother's initial allocation. In this experiment we show that testosterone levels in chicken eggs do not change with incubation period. A4 levels decrease between 3 and 5 days of incubation, which we cannot explain. Male eggs did not contain higher levels of testosterone or androstenedione than female eggs, in contrast to the data reported for another galliform species, the peacock. We conclude that it is unlikely that maternal androgens are a key factor in the avian sex determination mechanism.

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Keywords: Yolk androgens; Incubation period; Maternal hormones; Sex differences; White Leghorn

1. Introduction

Sex steroids of maternal origin can be found in the egg yolks of many oviparous species (Japanese quail, Adkins-Regan et al., 1995; Zebrafish, Canary, Cattle egret, American kestrel, Schwabl, 1993, 1996a,b, Schwabl et al., 1997, Sockman and Schwabl, 2000, Whittingham and Schwabl, 2002; turtles, Janzen et al., 1998; Zebrafish, Gil et al., 1999; Dark-eyed junco, Red-winged blackbird, Lipar et al., 1999; damselfish, McCormick, 1999; Painted turtle, Bowden et al., 2000; Black-headed gull, Eising et al., 2001; Lesser black-backed gull, Royle et al., 2001; Tree swallow, Whittingham and Schwabl, 2002). It has been suggested that yolk hormone levels correlate with circulating plasma hormone levels in the female (Canary, Schwabl, 1996a) and may vary adaptively with the species' reproductive strategy and social requirements (Cattle egret, Schwabl et al., 1997, Black-headed gulls: Eising et al., 2001; Groothuis and Schwabl, 2002). Maternal yolk hormones

influence the offspring's embryonic development (Eising et al., 2001), development of the hatching muscle (Red-winged blackbird, Lipar and Ketterson, 2000), begging behavior (Canary, Schwabl, 1996b, Eising and Groothuis, submitted), and nestling growth rate (Eising et al., 2001). Recently it has also been found that male and female producing eggs differ in the content of maternal androgens (Petrie et al., 2001). They found that Peafowl eggs hatching males have significantly higher contents of androstenedione and testosterone than eggs hatching females, whereas they contain lower levels of dihydrotestosterone and estradiol. This is of interest since maternal hormones may affect male and female offspring differently. It may also reflect a mother's differential investment in male and female offspring. In addition Petrie et al. (2001) have hypothesized that the concentrations of maternal steroids may play a role in the avian sex determining mechanism. However, the eggs in this study were analyzed at a relatively late stage of incubation (day 10 of the 28 days incubation period). This makes it difficult to interpret the results. The observed differences in steroid contents of male and female eggs may reflect not only the mothers' initial investment,

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but also the effect of differential development of male and female embryos. Especially in species with sexual size dimorphism, such as Peafowl used in the study by Petrie et al. there may be sex specific differences in the rate of development (Burke, 1994; Mitchell and Burke, 1995) and thereby in yolk utilization. This is of relevance since Lipar et al. (1999; Dark-eyed junco, Red-winged blackbird) and Möstl et al. (2001; Domestic chicken) showed that sex steroids of maternal origin are not evenly distributed through the egg yolk, but rather in concentric layers, with lower androgen concentrations in the most outer and most inner layers. Since it is generally believed that yolk usurpation starts in the outer layers of the yolk (Romanoff and Romanoff, 1960), it can be expected that concentrations of yolk hormones increase with yolk consumption and thus with incubation. This idea is confirmed by the results of Lovern and Wade (2001) in Green anoles, where testosterone levels increased with incubation. On the contrary, Elf and Fivizzani (2002) showed for the chicken that androstenedione, dihydrotestosterone, and testosterone levels decrease with incubation time and most steeply so shortly after incubation started. Unfortunately they did not include sex in their analyses. If the timing of utilization of the yolk layers is different for female and male embryos, sex differences in yolk hormone content may be found, that do not reflect the mother's initial allocation. On the other hand, mixing of the yolk from the onset of incubation onwards may counteract the uneven distribution of maternal hormones in the yolk.

Furthermore, in chicken embryos, endogenous production of steroids occurs as early as day 5 of development (Woods and Erton, 1978; Woods et al., 1975). The sex differences in yolk hormone content found by Petrie et al. (2001) may therefore be the consequence of a difference in endogenous production between males and females. Although it is not clear whether these embryonic hormones can enter the yolk, there is some evidence

for the presence of another embryonic steroid, corticosterone, in yolks of Tree lizard eggs (Jennings et al., 2000).

In this paper we present data of an experiment where we incubated chicken eggs for different numbers of days to investigate whether: (1) measurement of yolk androgens after the onset of incubation provides a good estimate for maternal hormone allocation. This is of relevance since hormone levels cannot be detected in freshly laid eggs if one also wants to reliably determine sex of the offspring. (2) Possible sex differences in yolk androgen levels are due to: (a) differences in maternal investment, (b) differences in the timing of yolk utilization or (c) differences in embryonic androgen production.

2. Materials and methods

2.1. Egg collection

Ninety eggs of 21 White Leghorn hens were collected over a 12 day period (November 9–21, 2001). Hens were housed in 3 groups of seven with a rooster in large outdoor aviaries with indoor roosting facility under natural daylight. Each group had food (Hendrix UTD, The Netherlands) and water available ad libitum. Within each aviary eggs could not be attributed to individual hens. Eggs were collected and weighed on the day of laying and marked per aviary (cage 1: 40 eggs, cage 2: 33 eggs, and cage 3: 17 eggs). The eggs were incubated in a commercial incubator at 37.5 °C and 60% humidity. After incubation eggs were stored at –20 °C for further analysis. Some freshly laid eggs were frozen instantly after collection. Overall, eggs were incubated for either 0 ($N = 10$), 3 ($N = 25$), 5 ($N = 15$), 7 ($N = 10$), 9 ($N = 10$) or 11 ($N = 20$) days. Later, eggs were defrosted, the yolks and embryos separated and weighed.

Table 1
Testosterone (T) and androstenedione (A4) levels (means \pm standard errors) for female and male producing eggs

Assay	1	1	2	2	2	2	2
Days of incubation	3	11	0	3	5	7	9
T females	1.91 \pm 0.17	2.00 \pm 0.11	1.16 \pm 0.20	1.75 \pm 0.51	1.00 \pm 0.11	1.81 \pm 0.54	1.11 \pm 0.17
(Mean \pm SE, N)	9	8	9	4	7	5	5
T males	1.96 \pm 0.18	2.02 \pm 0.12		1.12 \pm 0.39	0.71 \pm 0.17	0.79 \pm 0.19	1.52 \pm 0.17
(Mean \pm SE, N)	7	10		4	6	4	2
A4 females	45.59 \pm 2.98	30.62 \pm 3.22	23.40 \pm 5.58	19.82 \pm 3.18	11.88 \pm 0.83	14.22 \pm 1.54	15.01 \pm 3.78
(Mean \pm SE, N)	8	8	10	4	7	4	6
A4 males	53.86 \pm 6.46	30.56 \pm 3.02		21.90 \pm 3.34	12.52 \pm 3.01	12.31 \pm 1.56	31.35 \pm 13.56
(Mean \pm SE, N)	7	10		4	7	5	2
T sex differences	$p = 0.84$	$p = 0.94$		$p = 0.37$	$p = 0.18$	$p = 0.15$	$p = 0.23$
A4 sex differences	$p = 0.34$	$p = 0.96$		$p = 0.62$	$p = 0.72$	$p = 0.42$	$p = 0.13$

Data are presented separately for each assay and for the different incubation times. Test results of an independent T test are presented in the bottom rows for T and A4. (*) It should be noted that the samples marked by the asterisk in the 0 days incubation column are not of female but of undetermined sex.

Some eggs were unfertilized or insufficiently developed for the intended incubation time ($N = 6$) and therefore excluded from the analyses. In total 84 eggs were radioimmunoassayed for androgen content, of which one sample (age 7) failed. Final sample sizes per incubation time and sex are presented in Table 1 (see Section 3).

2.2. Radioimmunoassays

A competitive-binding radioimmunoassay (RIA) was used to determine androstenedione (A4) and testosterone (T) levels in the egg yolks. These RIAs were performed using the protocol as described by Wingfield and Farnar (1975) and Schwabl (1993) with slight modifications. Yolks were weighed and homogenized with an equal (1 ml/g yolk) amount of water. Around 150 mg of this mixture was used for analysis. Two thousand count per minute of tritiated androstenedione and testosterone (NEN, The Netherlands) were added to the yolk samples for calculation of recoveries. Samples were left to equilibrate overnight, after which they were extracted using two times 4 ml diethyl-/petroleum ether (70:30 vol.), dried under nitrogen and reconstituted in 1 ml of 90% ethanol. Samples were stored in the freezer at -20°C for at least 12 h and then spun down to remove neutral lipids. The samples were dried down again and reconstituted with 1 ml of 2% ethylacetate in 2,2,4-trimethylpentane before they were transferred to diatomaceous earth chromatography columns to elute each hormone fraction. A concentration of 2% ethylacetate was used for A4, 20% ethylacetate used for T fractions after 10% ethylacetate was used for extraction of the DHT fraction. A4 and T concentrations were measured by RIA, using hormone specific antibodies (Endocrine Science, USA) in two double radioimmunoassays. The first assay contained yolk samples that were either 3 or 11 days incubated (see Table 1). The second assay contained yolk samples that were 0, 3, 5, 7 or 9 days incubated. The average recovery rate for A4 was 63.3 and 43.7% for T. The inter-assay coefficient of variation was 17.6% for A4 and 14.0% for T, intra-assay variation coefficients were 12.8% for A4 and 9.6% for T.

2.3. Molecular sexing

DNA was extracted from approximately 1 μg of embryonic tissue using the Chelex resin-based technique (Walsh et al., 1991). Subsequently 2 μl of the extracted DNA solution was used for polymerase chain reaction (PCR) to amplify part of the CHD-W gene in females and the CHD-Z gene in both sexes (see Griffiths et al., 1998). The amplified products were separated on 2.5% agarose gels containing 0.005% ethidiumbromide for staining and visualized under UV light. Gender was assigned based on the presence of the CHD-Z gene only (males) or the presence of both genes (females).

2.4. Statistics

A4 yolk concentrations were not normally distributed and were therefore log transformed, after which they did show a normal distribution. T yolk concentrations did have a normal distribution and were not transformed. Sexes and incubation periods were evenly distributed over the three cages and therefore cage was not included in any of the models. In both assays eggs that had been incubated for 3 days were analyzed and there was an assay effect on the yolk hormone concentrations ($t_{22} = 7.69$, $p < 0.001$ for A4, $t_{23} = 1.85$, $p = 0.07$ for T) that may be due to differences in laying date or between individual hens (see Section 4). Therefore, results of both assays are discussed separately in the text. Since variances differed among incubation periods, a non-parametric anova was used to test the effect of incubation time. We used a full-factorial General Linear Model (GLM, SPSS) over all eggs to test the effect of sex (fixed factor) and incubation period (co-variate) on embryo weight, which was normally distributed. Fresh egg weight did not correlate with either yolk T ($t_{s9} = 0.30$, $p = 0.43$) or A4 ($r_{s10} = 0.15$, $p = 0.68$) levels and has not been included in any of the analyses.

3. Results

3.1. Sex differences

In neither of the assays and none of the incubation periods did T or A4 levels differ between the sexes for the different incubation times (Table 1).

Since sample sizes for the sexes are small and slightly skewed we combined the data of both assays for day 3 (the earliest day at which sex of the embryo's was determined) and for days 9 and 11 (the time period in which the Peafowl eggs were analyzed in the study by Petrie et al. (2001)). To correct for assay effects we standardized the data of each assay. There were still no significant sex differences at day 3 for either T ($t_{22} = 0.73$, $p = 0.47$, power = 0.75) or A4 levels ($t_{21} = -0.47$, $p = 0.47$, power = 0.73). Also later during incubation (day 9 and 11) there were no significant sex differences for T ($t_{23} = -1.40$, $p = 0.17$, power = 0.82) or A4 ($t_{24} = -1.70$, $p = 0.10$, power = 0.81).

3.2. Incubation

To explore whether and when T and A4 levels change between different stages of incubation, we analyzed the relation between yolk hormones and incubation time (Figs. 1A and B) in assay 2. In this assay both T and A4 levels showed unequal variances for the different incubation times, the data have been tested non-parametrically using a Kruskal Wallis Anova. There was no effect

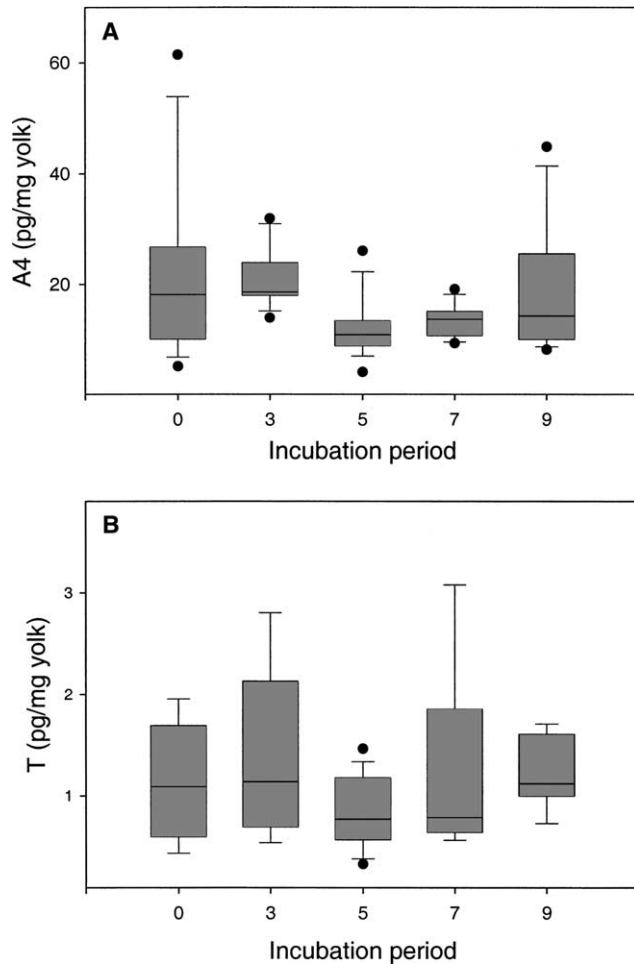


Fig. 1. (A) Boxplot for A4 concentrations (pg/mg yolk) with incubation period, (B) boxplot for T concentrations (pg/mg yolk) with incubation period. The boundary of the box closest to zero indicates the 25th percentile, a line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers above and below the box indicate the 90th and 10th percentiles. Dots mark the values in the 95th and 5th percentiles. Sample sizes for the consecutive incubation periods are 10, 8, 14, 9, and 8.

of incubation period on T levels ($\chi^2 = 3.36$, $df = 4$, $p = 0.50$). There was a significant effect of incubation time on A4 levels ($\chi^2 = 10.83$, $df = 4$, $p = 0.028$). Non-parametric post hoc tests indicated that yolk A4 levels differed significantly between days 3 and 5 ($z_{21} = -2.90$, $p = 0.003$) and days 3 and 7 ($z_{17} = -2.60$, $p = 0.008$). Although the difference between days 3 and 9 was not significant ($p = 0.16$) in this assay, the data from assay 1 confirm that there was a significant decrease in A4 levels over this period (days 3 and 11, $z_{34} = -3.93$, $p < 0.001$). Like in the previous assay T levels did not differ between these two days ($z_{35} = -0.96$, $p = 0.35$).

Since day 3 is the earliest moment embryos can reliably be sexed, it is important to know whether T and A4 levels differ between days 0 and 3. There were no such differences for either T ($z_{17} = -0.77$, $p = 0.48$) or A4 ($z_{18} = -0.53$, $p = 0.63$) levels.

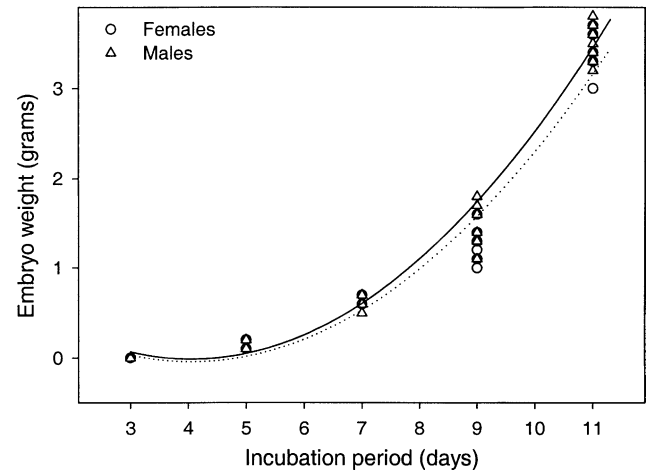


Fig. 2. Embryo weights (g) are plotted for males and females at the different stages of incubation. Sample sizes for males and females at the consecutive stages of incubation are: 10 and 7, 10 and 10, 5 and 4, 16 and 13, and 7 and 12.

3.3. Embryo weight

Secondary sex differences in yolk hormone levels could occur or could be masked if male and female embryos use their yolks differently. Fig. 2 shows the embryo weight for males and females plotted against incubation time. Embryo weight increased exponentially with incubation period squared ($p < 0.001$, $b = 0.52$), but there was no overall sex effect ($p = 0.46$) and only a trend for the interaction effect of sex and incubation time ($p = 0.075$). Male embryos ($b = 0.605$) were not heavier during the first 11 days of incubation than female embryos ($b = 0.497$).

4. Discussion

4.1. Sex differences in yolk steroid levels

Few studies have described the relation between yolk hormone levels and sex within individual eggs. Bowden et al. (2000) found an overall relation between clutch sex ratio and the ratio of maternal yolk estradiol and testosterone in freshly laid Painted turtle or Red-eared slider turtle eggs, but no direct association between sex of the embryo and hormone concentrations within the individual egg. In these chelonians sex is determined by incubation temperature (Bowden et al., 2001) while birds have genetic sex determination.

In birds, Petrie et al. (2001) showed sex specific hormone allocation for the Peafowl; eggs producing male offspring contained higher doses of the androgens A4 and T, whereas eggs producing females contained higher doses of estradiol and dihydrotestosterone. Following these results, they proposed that maternal steroids influence sex-chromosome segregation at the first meiotic

division and thus play a major role in the sex determining mechanism. Yet, the yolk hormone data were measured as far in development as day 10 in a 28 day incubation period. At this stage embryonic development and endogenous steroid production are well underway and may affect yolk androgen levels. Our data on eggs incubated for different time periods show no significant overall sex effect for A4 and T levels despite a reasonable power of the tests. In addition, at the earliest possible incubation stage for reliably sexing the embryos, day 3 (see below), there is no indication for a sex difference in either yolk T or A4 content. Chickens and peacocks are taxonomically closely related and both highly sexual dimorphic, and it seems unlikely that their sex determination mechanisms are very different. Technically, it should also be possible to sex freshly laid eggs, but there are so few embryonic cells at this stage that the risk of contamination with maternal tissue is very large. Moreover, freezing and subsequent thawing of the eggs to extract the total yolk makes it virtually impossible to find the blastodisc in freshly laid frozen eggs. The sex differences found in the peacock study might have been due to one of the following factors.

First, Galliformes male embryos start to produce testosterone in higher quantities than females from day 7 onwards (Rogers, 1995), which might be reflected in the yolk. Jennings et al. (2001) found some evidence for the presence of embryonic corticosterone in the yolks of Tree lizard eggs by day 25 of the 36 day incubation period. However, this has yet to be demonstrated for androgens, while we found no significant difference in the androgen concentrations of male and female eggs by day 11 in chicken eggs. Perhaps yolk samples in the Petrie et al. study contained some embryonic tissues such as blood vessels or blood, which could account for the sex difference they observed. A second possibility is differential growth rates between the sexes. There are several studies supporting this idea. In chickens, male embryos grow faster than female embryos, even if male and female eggs do not differ in size (Ahmad and Zamenhof, 1979; Mitchell and Burke, 1995). As males by day 9 have presumably having used up a larger proportion of the yolk mass, especially the outer layers that contain relatively low levels of androgens, differences in yolk steroid content between the sexes may appear. Our data show only a trend for the interaction effect of sex and incubation time on embryo weight. Despite this trend we have no indication that yolk consumption differed between the sexes since we found no sex differences in yolk hormone concentrations. Furthermore, the difference found by Petrie et al. by day 10 of incubation is much larger than could be explained by differential yolk consumption until that time. Thirdly, the fact that Petrie et al. (2001) did find a sex effect on yolk hormone levels may be explained by the influence of the social structure in the aviaries. Sex specific androgen levels in

eggs, incubated for 3 days, have recently been found in relation to the social status of a hen (Müller et al., 2002). If in the peacock study eggs were collected not randomly with respect to such social factors, this may have confounded the interpretation. Also in our study we did not take social rank into account. It would be interesting to know whether the sex differences found in the Peacock study were already present from day 3 onwards and really indicate differential investment in sons and daughters.

Since we measured only A4 and T (the hormones present in high amounts in male egg in the study by Petrie et al.), our data do not rule out the possibility that maternal yolk hormones or their ratio are involved in sex determination. Yet, we did not observe sex specific androgen deposition in chicken eggs that was so clearly present in the Petrie et al. study.

4.2. Yolk steroids and incubation time

Recently, another study described the effect of incubation time on yolk hormone levels in the chicken (Elf and Fivizzani, 2002). That study and the present study report similar initial levels of A4 (24.06 resp. 23.4 pg/mg yolk), whereas T levels are only slightly different and low in both studies (0.46 resp. 1.16 pg/mg yolk). This facilitates the comparison of both studies. Elf and Fivizzani showed that A4, DHT, and T levels decreased significantly with incubation time, whereas E2 levels increased considerably after 14 days of incubation. In contrast to our results, A4, DHT, and T levels already dropped significantly between days 0 and 1 of incubation. The authors offer no explanation for this very early decrease. Perhaps the non-incubated eggs had higher levels since they were immediately frozen after collection in contrast to the other eggs, which were stored for a certain amount of time prior to incubation.

There were no differences in either yolk T or A4 levels between days 0 and 3 in our study. Day 3 is the earliest age at which the embryos can be sexed and our study suggests that steroid levels measured on day 3 are a reliable indicator for the mother's initial hormone allocation in relation to sex.

In assay 1 A4 levels decreased significantly between days 3 and 11. In assay 2 there were indications that A4 levels also decreased after day 3, while T levels also dropped slightly, but not significantly so, after day 3 of incubation. Interestingly, a similar drop at the same time was reported for DHT and T in the Elf and Fivizzani study. At this stage the embryo is still very small and only a small portion of the yolk (average 1.89 g, 11.5%) is used up between days 3 and 5. Moreover, if especially the outer yolk layers with relatively low androgen levels are being consumed during embryonic development one would expect androgen levels to increase rather than decrease, unless there is extensive mixing of the yolk or a

water influx from the albumen. In Green anoles, Lovern and Wade (2001) found such an increase in yolk T levels over the whole incubation period. Nor Elf and Fivizzani, nor we can explain this decrease at this point of time. Further studies, using larger sample sizes in a variety of species are clearly needed.

Unfortunately, the inter-assay variation was relatively large, possibly due to effects of laying date, inter-hen variation and other factors for which we could not control. Also the intra-assay variation was relatively high, but not usually so. Elf and Fivizzani report comparable levels of inter- and intra-assay variation for their chicken assays. Our recovery rates are somewhat lower than in the study by Elf and Fivizzani, but not usually low as compared to other studies. Yet, the combination of relatively low recovery rates, high coefficients of variation, and small sample sizes may obscure some effects of incubation time and sex. However, with respect to all our questions, both assays show for both hormones similar results: much higher A4 than T levels, no sex effects, and a slight decrease in A4 but not in T levels after day 3.

We conclude that yolk androgen levels seem to be affected by incubation only after several days. Thus, a mother's hormonal investment into her offspring in relation to the offspring's sex can be reliably determined before day 3 of incubation. We have found no evidence in the chicken that maternal yolk androgens are allocated in a sex specific way. We found no evidence for sex specific growth differences and there was no indication that endogenous androgen production was reflected in the egg yolks.

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References

Adkins-Regan, E., Ottinger, M.A., Park, J., 1995. Maternal transfer of estradiol to egg yolks alters sexual differentiation of avian offspring. *J. Exp. Zool.* 271, 466–470.

Ahmad, G., Zamenhof, S., 1979. The effect of progesterone on brain and body growth of chick embryos. *Growth* 43, 58–61.

Bowden, R.M., Ewert, M.A., Nelson, C.E., 2000. Environmental sex determination in a reptile varies seasonally and with yolk hormones. *Proc. R. Soc. Lond. B* 267, 1745–1749.

Bowden, R.M., Ewert, M.A., Lipar, J.L., Nelson, C.E., 2001. Concentrations of steroid hormones in layers and biopsies of chelonian egg yolks. *Gen. Comp. Endocrinol.* 121, 95–103.

Burke, W.H., 1994. Sex-differences in weight of turkey embryos. *Poult. Sci.* 73, 749–753.

Eising, C.M., Groothuis, T.G.G. (submitted), Maternal androgens enhance begging behaviour in Black-headed gull chicks, *Anim. Behav.*

Eising, C.M., Eikenaar, C., Schwabl, H., Groothuis, T.G.G., 2001. Maternal androgens in Black-headed gull (*Larus ridibundus*) eggs: consequences for chick development. *Proc. R. Soc. Lond. B* 268, 839–846.

Elf, P.K., Fivizzani, A.J., 2002. Changes in sex steroid levels in yolks of the Leghorn chicken, *Gallus domesticus*, during embryonic development. *J. Exp. Zool.* 293, 594–600.

Gil, D., Graves, J., Hazon, N., Wells, A., 1999. Male attractiveness and differential testosterone investment in zebra finch eggs. *Science* 286, 126–128.

Griffiths, R., Double, M.C., Orr, K., Dawson, R.J.G., 1998. A DNA test to sex most birds. *Mol. Ecol.* 7, 1071–1075.

Groothuis, T.G.G., Schwabl, H., 2002. The influence of laying sequence and habitat characteristics on maternal yolk hormone levels. *Funct. Ecol.* 16, 281–289.

Janzen, F.J., Wilson, M.E., Tucker, J.K., Ford, S.P., 1998. Endogenous yolk steroid hormones in turtles with different sex-determining mechanisms. *Gen. Comp. Endocrinol.* 111, 306–317.

Jennings, D.H., Weiss, S.L., Moore, M.C., 2000. Ontogenetic changes in embryonic yolk steroid content in tree lizards: transfer of hormones from the developing embryo to the yolk? *Am. Zool.* 40, 1075–1076.

Lipar, J.L., Ketterson, E.D., 2000. Maternally derived yolk testosterone enhances the development of the hatching muscle in the red-winged blackbird *Agelaius phoeniceus*. *Proc. R. Soc. Lond. B* 267, 2005–2010.

Lipar, J.L., Ketterson, E.D., Nolan Jr., V., Casto, J.M., 1999. Egg yolk layers vary in the concentration of steroid hormones in two avian species. *Gen. Comp. Endocrinol.* 115, 220–227.

Lovern, M.B., Wade, J., 2001. Maternal plasma and egg testosterone concentrations during embryonic development in Green anoles (*Anolis carolinensis*). *Gen. Comp. Endocrinol.* 124, 226–235.

McCormick, M.I., 1999. Experimental test of the effect of maternal hormones on larval quality of a coral reef fish. *Oecologia* 118, 412–422.

Mitchell, R.D., Burke, W.H., 1995. Genotype and sexual influences on growth and muscle development of chicken embryos. *Growth Dev. Aging* 59, 31–44.

Möstl, E., Spendier, H., Kotrschal, K., 2001. Concentration of immunoreactive progesterone and androgens in the yolk of hens' eggs (*Gallus domesticus*). *Wien Tierarztl. Monat.* 88, 62–65.

Müller, W., Eising, C.M., Dijkstra, C., Groothuis, T.G.G., 2002. Social dominance and maternal investment: yolk androgens and offspring sex in domestic chickens. *Proc. R. Soc. Lond. B* 269, 2249–2255.

Petrie, M., Schwabl, H., Brande-Lavridsen, N., Burke, T., 2001. Sex differences in avian yolk hormone levels. *Nature* 412, 498.

Rogers, L.J., 1995. The development of brain and behaviour in the chicken. CAB International, The University Press, Cambridge, UK.

Romanoff, A.L., Romanoff, A.J., 1960. In: The avian egg. Chapter 4: formation. John Wiley & Sons, Inc, New York, pp. 175–255.

Royle, N.J., Surai, P.F., Hartley, I.R., 2001. Maternally derived androgens and antioxidants in bird eggs: complementary but opposing effects? *Behav. Ecol.* 12, 381–385.

- Schwabl, H., 1993. Yolk is a source of maternal testosterone for developing birds. *Proc. Natl. Acad. Sci. USA* 90, 11446–11450.
- Schwabl, H., 1996a. Environment modifies the testosterone levels of a female bird and its eggs. *J. Exp. Zool.* 276, 157–163.
- Schwabl, H., 1996b. Maternal testosterone in the avian egg enhances postnatal growth. *Comp. Biochem. Physiol.* 114, 271–276.
- Schwabl, H., Mock, D.W., Gieg, J.A., 1997. A hormonal mechanism for parental favouritism. *Nature* 386, 231–231.
- Sockman, K.W., Schwabl, H., 2000. Yolk androgens reduce offspring survival. *Proc. R. Soc. Lond. B* 267, 1451–1456.
- Whittingham, L.A., Schwabl, H., 2002. Maternal testosterone in tree swallow eggs varies with female aggression. *Anim. Behav.* 63, 63–67.
- Wingfield, J.C., Farner, D.S., 1975. The determination of five steroids in avian plasma by radioimmunoassay and competitive protein-binding. *Steroids* 26, 311–327.
- Woods, J.E., Ertton, L.H., 1978. The synthesis of estrogens in the gonads of the chick embryo. *Gen. Comp. Endocrinol.* 36, 360–370.
- Woods, J.E., Simpson, R.M., Moore, P.L., 1975. Plasma testosterone levels in the chick embryo. *Gen. Comp. Endocrinol.* 27, 543–547.